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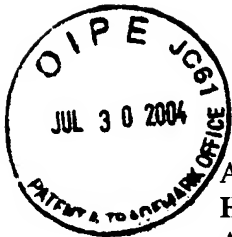
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **David Lewis,**
Hans Herweijer, James E. Hagstrom,
Aaron Loomis, Jon A. Wolff,

Serial No.: **10/007,448**

Examiner: **Terra C. Gibbs**

Filed: **11/07/2001**

Group Art Unit: **1635**

For: **Inhibition Of Gene Expression By Delivery Of Small Interfering RNA To
Post-Embryonic Animal Cells *In vivo***

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. 1.131

Dear Sir:

I, David L. Lewis, hereby declare as follows:

1. I am an inventor of the processes found in the captioned patent application.
2. Photocopies of pages from my, David Lewis', personal laboratory notebook show experimental evidence of delivery of short polynucleotides to animal cells.
3. It is known to me that the process performed in the notebook pages results in the delivery of expression inhibiting polynucleotides to animal cells as described in my specification.
4. The process was conceived and performed prior to the effective date of the Office Action prior art references indicating delivery of expression inhibiting polynucleotides.
5. Development of this process occurred with due diligence from the date of conception to the filing of the application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.


David L. Lewis, Ph.D.


Date

From Page No. _____

Purpose: Determine if conjugation of DL57:DL58 siRNA with pMIR48 inhibits Luc+ expression

Materials:

pMIR48
DL57:DL58 siRNA 40 mM 0.53 µg/µl 7/9/01

Methods:

Group 1 pMIR48 (10 µg) + 0.5 µg DL57:DL58 2 animals each
Group 2 " + 5 µg DL57:DL58 "
Group 5 " + 0 µg "

Inject by high pressure tail vein injection in TCR mice
Homogenize liver measure Luc+ activity on Day 1

Results:

Luciferase Assay - Liver thaws

hp tail vein
liver homogenate 20hr
10µg pMIR48 all
assay 10µl normal Luc+ assay

DL57:DL58 siRNA (µg)	LU	AVE	undiluted LU	normalized
0	undil 21288848 1:10 4139850	33804016 9485125	6812386 68123875	1
0.5	undil 15414087 1:10 2183854	12688318 2030952	2107403 21074030	0.3093487
5	undil 3337360 1:10 ND	4820293 ND	4449560	0.0853157

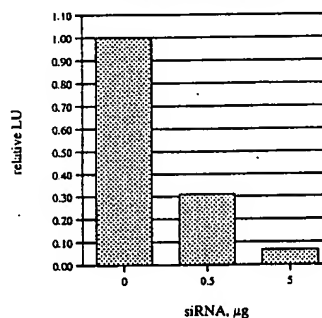
MEASUREMENT	ROUTINE
19 AUG 01 10:03	V.2.03
PROTOCOL NO. : 8	NAME : 10
VOLUME INJ. 1 (ul) : 50	
VOLUME INJ. 2 (ul) : 50	
SEQUENCE OF INJECTIONS : 1-2	
DELAY TIME INJ 1/INJ 2 (s) : 2.0	
MEASURE BACKGROUND : NO	
DELAY LAST INJ./MEAS. (s) : 0.5	
MEASURING TIME (s) : 10.0	

COMMENT :

SAMPLE RLU

1	15414087	} grp 1 0.5 µg
2	2183854 10x	
3	12688318	} grp 2 5 µg
4	2030952 10x	
5	3337360	} grp 5 0 µg
6	4820293	
7	21288848	
8	4139850	
9	33804016	
10	9485125 10x	

pMIR48/siRNA hp tail 8/9/01



pMIR48, 10µg all

Results indicate substantial knock-down of Luc+ activity by siRNA in liver. Repeat exp with control siRNA to show sequence specificity. Also try injecting pMIR116 & pMIR122 with siRNA against pMIR116 to provide more evidence of specificity.

To Page No. _____

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

TITLE hp tail vein injection of pMIR116/pMIR122 + S. RNABook No. 180From Page No.

Purpose: Determine if S. RNA is specific to Lact in the liver after
hp tail vein delivery

Materials:

pMIR116

pMIR122

DL57:DL58 S. RNA 40 mM 0.53 mg/L 7/9/01

Pronega Dual Luciferase kit

Methods:

Group 1 pMIR116 (10ng) + pMIR122 (1ng) + 0.5 mg siRNA

Group 2 " " + 5 mg S. RNA

Group 3 " " w/o S. RNA

Group 4 pMIR116 (10ng)

Group 5 pMIR122 (1ng)

Inject by hp tail vein in ICR mice
Homogenize liver in Lys lysis Buffer

Dilute 1/100 in 1x PLB

Assay 10x with Dual Luciferase kit

Results:

Livers looked

good at

harvest

Inhibition

specific to

Lact target

R. Luc not

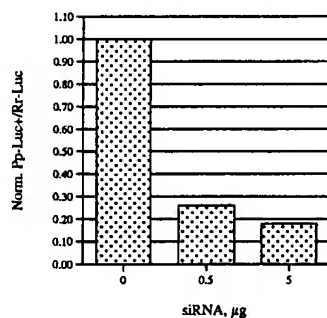
affected.

siRNA specific

not occurring

in liver.

pMIR116+pMIR122+siRNA hp tail 8/14/01

pMIR116 10µg
pMIR122 1µg

8/14/01
hp tail vein
liver homogenate
Dual Luciferase assay, 10 µl of 1/100 dilution in PLB
10 µg pMIR116/1 µg pMIR122

#	1	2	average	total average	Relative #	Normalized
#8252						
0.5 µg siRNA						
Luciferase	321860	324268	323064	448307	0.47	0.26
Renilla	545858	574980	560419	656373		
#8225						
0.5 µg siRNA						
Luciferase	573550		573550			
Renilla	1358326		1358326			

#	1	2	average	total average	Relative #	Normalized
#8226						
5 µg siRNA						
Luciferase	332135	349741	340938	220181	0.32	0.16
Renilla	945021	1031082	988052	691971		
#8221						
5 µg siRNA						
Luciferase	99424		99424			
Renilla	395891		395891			

#	1	2	average	total average	Relative #	Normalized
#8224						
0 µg siRNA						
Luciferase	731758	814849	773304	614692	1.78	1.00
Renilla	457849	507597	482723	345955		
#8241						
0 µg siRNA						
Luciferase	469566	442595	456081			
Renilla	219182	198180	208186			

#	1	2	average	total average	Relative #	Normalized
#8262						
10 µg pMIR116 only						
Luciferase	2181219		2181219	1505354	379.37	213.52
Renilla	4920		4920	3968		
#8245						
10 µg pMIR116 only						
Luciferase	829489		829489			
Renilla	3016		3016			

#	1	2	average	total average	Relative #	Normalized
#8261						
1 µg pMIR122 only						
Luciferase	12237		12237	6824	0.02	0.01
Renilla	793405		793405	651220		
#8264						
1 µg pMIR122 only						
Luciferase	1410		1410			
Renilla	508034		508034			

No effect on R. Luc suggests interferon
pathway not activated in liver by S. RNA

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

[Signature]

8/7/01

[Signature]

8/14/01

From Page No.

Purpose: Determine if RNAi works in different organs in mice

Materials:

pM12116 3.79 ug/l make 1 ug/l dilution in Endofree TE 131.9 n + 368.1 n TE
pM12122 2.46 ug/l make 0.1 ug/l dilution " 20.3 n + 479.7 n TE
DL57:DL58 siRNA 40 nM 0.53 ug/l duplex 7/8/01 make 0.5 ug/l dilution
DL64 0.1 nmol/l 0.67 ug/l RNase free H₂O
DL65 0.1 nmol/l 0.68 ug/l "

Methods:

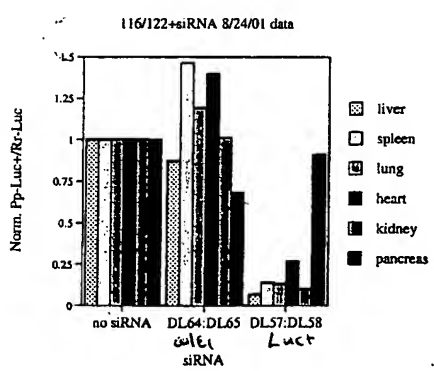
Animal DL64 and DL65

DL64	100 n	✓	10 nmol		Prog #19	94°C	2'
DL65	100 n	✓	10 nmol	⇒ 40 nM duplex		90°C	1'
1M Tris pH8	12.5 n	✓	50 nM final	0.54 ug/l		20°C	1' min
5M NaCl	5 n	✓	100 nM final			40°C	∞
H ₂ O	32.5 n	✓		make 0.5 ug/l dilution			
	250 n			for ACh			

Animal more DL57 and DL58

DL57	100 n	✓	10 nmol	Prog #19	⇒ 40 nM
DL58	100 n	✓	10 nmol		0.53 ug/l
1M Tris pH8	12.5 n	✓	50 nM	make 0.5 ug/l dilution	
5M NaCl	5 n	✓	100 nM	for ACh	
H ₂ O	32.5 n	✓			
	250 n				

Group 1 pM12116(10 ng) + pM12122(1 ng) + DL57:DL58 siRNA (5 ng)
Group 2 " " + DL64:DL65 siRNA (5 ng)
Group 3 " " no siRNA



Results: 93% 86% 87% 73% 90% 0% inhibited
See RNAi in liver, spleen, lung, heart, kidney.
In pancreas no RNAi is observed. Degradation by pancreatic enzymes? or rather only 8% inhibited.
NO RNAi observed with DL64:DL65 directed at plasmid backbone (colE1 ori).

To Page No.

Witnessed & Understood by me,

Date 9/7/01

Invented by
Recorded by

Date 8/22/01

TITLE Injecting Morpholino: oligo into mice + DNA

From Page No. _____

Purpose: Investigate whether morpholino annealed to DNA oligo bridge enhances morpholino uptake in vivo using high pressure tail vein injection.

Materials: see p. 12

- FITC - morpholino control oligo - GeneTools (DL012)
1,000 pmol/l 8.3 µg/l in TE
- unlabeled DNA oligo DL010
100 pmol/l 0.97 µg/l in TE
- Cy3 labeled DNA oligo DL011
100 pmol/l 1.01 µg/l in TE
- pMIR48 endfree Max: 2.90 µg/l in H₂O 9/14/00

Methods:

Anneal oligos in TE (10 mM Tris, 0.1 mM EDTA) + 150 mM NaCl

#		Morpholino	DL010	DL011	TE	NaCl	tot vol	µg/l	Run on gel	need
1	2.5x	12.6n ^{12,600 pmol} 104.8 µg	12.6n ^{12,600 pmol} 122 µg	-	4.2n ^{4,200 pmol} 4.2n	4.2n ✓	~140n	1.62	(3.5x)	8
2	2x	3.6n ^{3,600 pmol} 30 µg	-	-	3.2n ✓	1.2n ✓	40n	0.75	(1x)	2
3	2x	-	-	3.6n ^{3,600 pmol} 36.4 µg	2.8n ✓	1.2n ✓	40n	0.91	(1x)	2
4	1x	-	3.6n ^{3,600 pmol} 35 µg	-	2.8n ✓	1.2n ✓	40n	0.97	(1x)	1

Anneal 94°C 2' 19
80°C
↓ 1°/degree
10°C
4°C ∞

Ligate 40n of morpholino oligo annealed
✓ 40n DNA (64.8 µg)
✓ 4n 10x Buffer ligase
2n Ligase (400 uln)
40n

ligate 16°C 1h

Label 60 µg pMIR48 w/ Label IT Cy3

dH₂O 189.3 ✓

10x A 30n ✓

pMIR48 20.7 ✓ (60 µg)

Cy3 Label IT 60n
300n

incubate 37°C, 1 hr

start 2:15 pm

end 3:15 pm

Clean up ~~on 6 columns~~ ^{on 9/27/00}

by EtOH ppt

Wash w/ 70% EtOH

Resuspend 30n → 2 µg/l

To Page No. 56

Witnessed & Understood by me,

Herweyer

Date

10/10/00

Invented by

Recorded by

Dee

Date

9/21/00

From Page No. 55

Injections to do								Morpholino
#animals			vol	mg	code	tot vol		mg/animal
3	Morpholino: oligo	1	40 μ	65	DL1	120 μ ✓		30
3	morpholino: oligo ligated	1-4	40 μ	65	DL2	120 μ ✓		30
2	Morpholino	2	40 μ	30	DL3	80 μ ✓		30
2	oligo Cy3	3	40 μ	36.4	DL4	80 μ ✓		-
	pMIR248-Cy3			30			morph DNA	-
2	morpholino: oligo pMIR248-Cy3	1	55 μ	95	DL5	(80 μ + 30 μ) \times 2 = 30		30
				(65+30)				

morph oligo Cy3 oligo morph oligo morph oligo ligated
Gel - 3% Agarose H/S w/ EtBr (10 μ in 80 μ of loading solution)

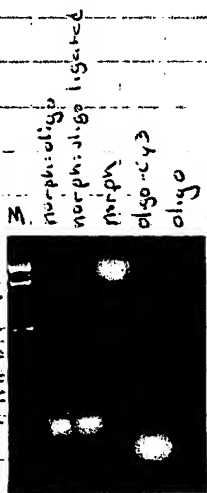
M	DL3	DL4	#4	DL1	DL2	
	2.67	2.20	2.3 μ	1.23	1.41	vol (2 mg total each)
	6.33	6.8	6.7	7.77	7.59	TNE 10 μ M Tris 150 μ M NaCl 0.1 M EDTA
1x						10x Buffer

Load - M DL1 DL2 DL3 DL4 4 2 μ 60V start: 10:45a
end:

Results:

- Morpholino alone barely migrates into the gel - uncharged
- morpholino: oligo annealed results in all morpholino being taken into the gel. Size range - 60 - 200 bp, although hard to estimate size of this copolymer as only one of the strands is charged. The fact that the morpholino is taken into the gel indicates that it is paired w/ the oligo. Most is probably annealed to 1 oligo or maybe 2. Longer oligomers are probably much less stable.

Able to see green fluorescence of morpholino and red fluorescence of oligo Cy3 under 302 nm light.



To Page No. 69

Witnessed & Understood by me.

Herweyer

Date

12/22/00

Invented by

Recorded by

DA

Date

9/21/00

From Page 56

Collect livers 24 hrs later

Cut sections (10 μ m)

Fix in 1% formaldehyde: PBS RT 15'

Wash 3x5' PBS

incubate in 1:10,000 TB Pro-3 20'

Rinse in PBS

Mount

Get 1 slide/animal in each group - will cut others on Mon. if necessary

3 OLC1 morph: oligo

3 OLC2 morph: oligo 1.5x2x2

high bkgnd

2 OLC3 morph

try MeOH step

2 OLC4 oligo Cy3

label all as: OLC1, OLC2, OLC3, OLC4

2 OLC5 morph: oligo + pMIRCy3

OLC1, slide 2 1-2-3
OLC2, slide 2 1-2-3
OLC3, slide 3 1-3

Collect

Get remaining slides Mon

Fix tissue w/ 1% formaldehyde as above

Wash 1x5' PBS

Place in 100% MeOH 10'

Wash 3xPBS 5'

Incubate in 1:40,000 TB Pro-3 20' (1.75% in 70 mls)

Injections

Group 1 (OLC1) 1 - great inj

2 - not great

3 - not great

Group 2 (OLC2) 1 - great

2 - great

3 - great

Group 3 (OLC3) 1 - great

2 - great

Group 4 (OLC4) 1 - great

2 - great

Group 5 (OLC5) 1 - great

To Page No. 70

Investigator's Name

Date

Investigator's Name

Date

Jernigan

10/10/00

Investigator's Name

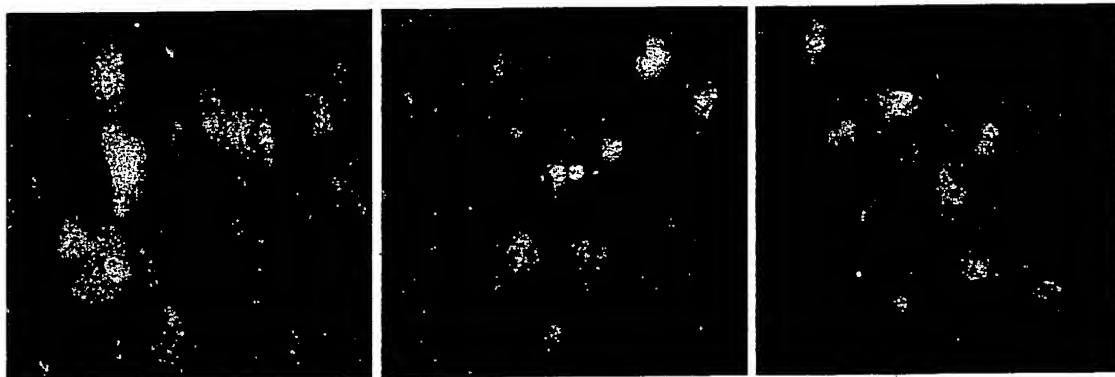
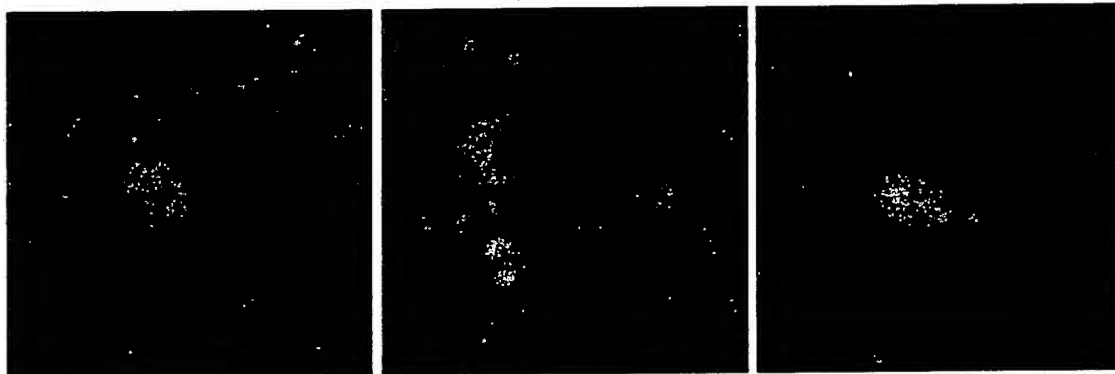
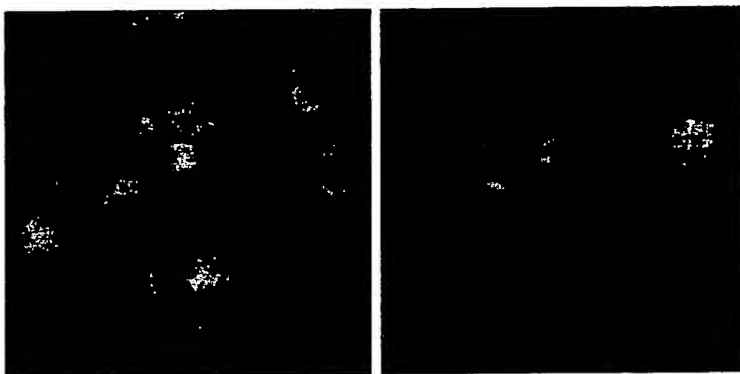
10/10/00

9/24/00

From Page No. 69

Results:

morpholino

morpholino
:DNA oligomorpholino
:DNA oligo
ligatedmorpholino
:DNA oligo
+ plasmid

42.71

Hermeyer

10/10/00

Prepared by

Dau

10/2/00

TIT-3 Injecting morpholinos oligo into mice cells

Page No. 70Conclusions:

1. Morpholinos alone able to enter cells! Pattern is diffuse cytoplasmic + nuclear staining. This is unexpected. Route of uptake?
2. Morpholinos + oligo able to enter cells as well. Seen to be fewer cells than with morpholinos alone, but small sample size makes conclusions difficult. No difference if DNA strand is treated with ligase.
3. When morpholinos + oligo + Cy3 labeled DNA (plasmid) are injected simultaneously, both enter same cells and to similar relative amounts. Plasmid DNA can be seen as punctate nuclear staining in some cells, punctate cytoplasmic + nuclear staining in other cells, and cytoplasmic only in a minority of cells. In some nuclei w/ plasmid DNA + morpho oligo, both seen to be located in same sub-nuclear location as punctate staining.

Investigate:

1. Time course of uptake
2. inhibition of gene expression
3. low pressure injections

To Page No. _____

Witnessed & Undertaken by me,

Hewit

Date

10/10/00

Initiated by

Recorded by

Dae

Date

10/2/00